·Basic Research·

Study on the optical property and biocompatibility of a tissue engineering cornea

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Abstract

• AIM: To study the optical property and biocompatibility of a tissue engineering cornea.

• METHODS: The cross-linker of N-(3-Dimethylaminoropyl) -N'ethylcarbodiimide hydrochloride (EDC)/ N-Hydroxysuccin-imide (NHS) was mixed with Type I collagen at 10% (weight/volume). The final solution was molded to the shape of a corneal contact lens. The collagen concentrations of 10%, 12.5%, 15%, 17.5% and 20% artificial corneas were tested by UV/vis-spectroscopy for their transparency compared with normal rat cornea. 10-0 sutures were knotted on the edges of substitute to measure the corneal buttons's mechanical properties. Normal rat corneal tissue primary culture on the collagen scaffold was observed in 4 weeks. Histopathologic examinations were performed after 4 weeks of in vitro culturing.

• RESULTS: The collagen scaffold appearance was similar to that of soft contact lens. With the increase of collagen concentration, the transparency of artificial corneal buttons was diminished, but the toughness of the scaffold was enhanced. The scaffold transparency in the 10% concentration collagen group resembled normal rat cornea. To knot and embed the scaffold under the microscope, 20% concentration collagen group was more effective during implantation than lower concentrations of collagen group. In the first 3 weeks, corneal cell proliferation was highly active. The shapes of cells that grew on the substitute had no significant difference when compared with the cells before they were moved to the scaffold. However, on the fortieth day, most cells detached from the scaffold and died. Histopathologic examination of the primary culture scaffold revealed well grown corneal cells tightly attached to the scaffold in the former culturing.

• CONCLUSION: Collagen scaffold can be molded to the shape of soft contact corneal lens with NHS/EDC. The biological stability and biocompatibility of collagen from animal species may be used as material in preparing to engineer artificial corneal scaffold.

• KEYWORDS: Tissue engineering; Collagen cross-linking scaffold; Primary culture in vitro; Optical property; Biocompatibility
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INTRODUCTION

Corneal disease is a major cause of blindness, second only to cataracts [1]. Once the cornea is injured, neovascularization, scarring, and edema may follow. Injury may render the cornea to lose it transparency, especially in the setting of irreversible corneal scarring. With increase of infectious disease agents such as HIV and hepatitis, as well as the field of refracttive surgery, global cornea donor supply for transplantation may become insufficient. The research of donor cornea alternatives is significant[2].

Since the 18th century, scientists have completed extensive study in the field of Keratoprosthesis (Kpro) [3]. A variety of compounds have been used in Kpro research, including silicone rubber[4], expanded polytetrafluoroethylene (ePTFE)[5], polymethylmethacrylate (PMMA) [6], and hydrophilic polymer poly (2-hydroxyethyl methacrylate) [7]. However,

these Kpros are foreign bodies for recipients and it uses for surgery are limited. Moreover, complications are the main reasons for the transplant failures, to include examples such as tissue necrosis and melt, soft contact lens loss, inflammation, retroprosthetic membrane, infectious endophthalmitis, sterile uveitis-vitritis, glaucoma, retinal detachment [3]. Therefore, tissue-engineering cornea is an attractive alternative to foregin bodies if biocompatibility is successful. As early as 1982, Friend *et al* [8] had cultured epithelial cells on denuded corneal stroma in vitro. Since 90% of corneal tissue is composed of collagen fibers, scientists tried to use collagen gel [9-13], oral mucosal epithelium [14], acellular porcine cornea [15-19], chitosan [20], etc. as scaffolds for corneal cell growth. The "air-liquid interface" method [21-27] has been used to reconstruct the three-dimensional matrix.

Griffith *et al* [28] has completed extensive work in researching biocompatible artificial cornea. They have successfully implanted substitutes into rabbit and porcine corneas, however, cell lines were used in most of their research. This study focuses on simulating biocompatibility and optical properties of a cornea *in vitro*.

MATERIALS AND METHODS

Materials Type I Collagen (C9879-25G, Sigma-Aldrich, USA), NHS (HB0526-25g, BBI, Canada), EDC (E7750-5g, Sigma-Aldrich, USA), HCL in Analytical reagent (Shenyang Minlian chemical Co., Ltd, China), Dulbecco's Modified Eagle Media (DMEM, High glucose, Thermo Scientific Hyclone, China), 0.25% Trypsin (Thermo Scientific Hyclone, China), Phosphate Buffered Saline (PBS, 1X, Thermo Scientific Hyclone, China), Fetal Bovine serum (FBS, Thermo Scientific Hyclone, China), Penicillin streptomycin (PS, 100X, Thermo Scientific Hyclone, China), Epidermal Growth Factor (EGF, Peprotech, China). SD Rats were purchased form Laboratory Animal Department of China Medical University, all the animals were treated according to the Association for Research in Vision and Ophthalmology and World Medical Association Declaration of Helsinki tenets. UV-1800 (Shimadzu, Kyoto, Japan).

Methods

Creating the scaffold The substitute contained 20% (wt/wt) type I collagen, and crosslinking solution of EDC/NHS (EDC: NHS=2:1 in molar ratio) was calculated at 10% (wt/vol). The type I collagen was dissolved in an HCL solution (PH) at 50℃ for 2 hours. During this time, the solution remained on a vortex machine for 10 minutes at 30 minute intervals. The collagen solution was then adjusted to pH 5±0.5 with 10.0M aqueous NaOH. The EDC/NHS cross-linker was mixed in the following solution by using a syringe mixing technique at 10 000rpm for 30 seconds to remove air bubbles. Immediately, the final solution was injected to culture dishes in corneal contact lens molding. The dishes were cured at 21℃ for 24 hours and 37℃ for 24 hours under 100% humidity. Then, the implant was washed in phosphate-buffered saline (PBS) three times and stored in PBS containing 5% (vol/vol) penicillin/streptomycin combination and 10% (vol/vol) gentamicin at 4℃ to maintain sterility[28, 29].

Characterization of collagen samples Transparency measurements: To find a concentration that most replicated corneal optical and mechanical properties, corneal buttons in concentrations of 10%, 12.5%, 15%, 17.5% and 20% were used. The substitutes shaped at 500±50μm thick in a specialty mold were tested for transparency by UV/vis-spectroscopy (UV-1800) to narrow spectral regions, respectively, at 450, 500, 550, 600, 650 and 700nm.

Mechanical properties To measure the application for implantation surgery, 10-0 sutures (33μm) were used for operating knots under the microscope in vitro to embed the edges at gradient concentrations described previously. Evaluation standards were assessed by examining for shearing and tearing[30].

Corneal tissue in primary culture on collagen scaffold SD rats were killed by wringed their necks. Rat corneas were extracted by cutting along their limbus under optical microscope within 12 hours after they were killed. The corneal buttons were washed twice with the phosphate-buffered saline (PBS) to remove the blood and remaining iris and each cornea were cut into 4 blocks in sector with scissors and immediately put into culture disks which were structured with corneal shape collagen and overspread by 200μL Dulbecco's Modified Eagle Media (DMEM) with 10% FBS and penicillin/streptomycin (PS) in advance. Make sure each corneal epithelium was up-side and the limbus of the cornea was closed to the collagen button. The culture dishes were put into carbon dioxide incubator at 37℃ with 5% CO_2 for 2 hours. After the corneal tissue adhered the inner face of the culture dish tightly, added the culture solution (DMEM+10% FBS+1× PS+0.01% EFG) into 3mL. Each dish was added 10μL gentamycin at the first time to protect the fungal contamination. And the culture solution was changed every three days.

RESULTS

Collagen Scaffold Corneal substitutes are shaped similar to soft corneal contact lens (Figure 1).

Transparency Measurements The optical property of corneal buttons in different concentrations had no obvious differences in appearance, and their collagen percentage could not be distinguished on inspection (Figure 2A). The 10% concentration group was most resembled normal rat cornea (Figure 2B). However, the results showed the transmittance of the scaffold had increased with the decrease of collagen percentage (Figure 2C).

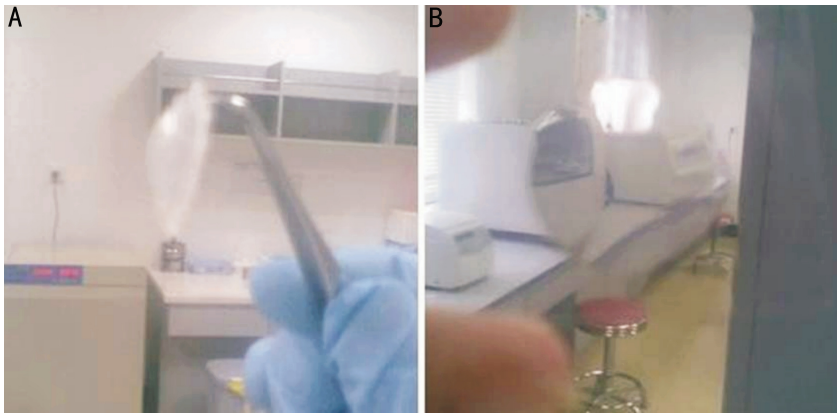

Figure 1 Digital images of collagen scaffold: 500±50μm thickness, the concentration of collagen was 20% A: Side view; B: Front view.

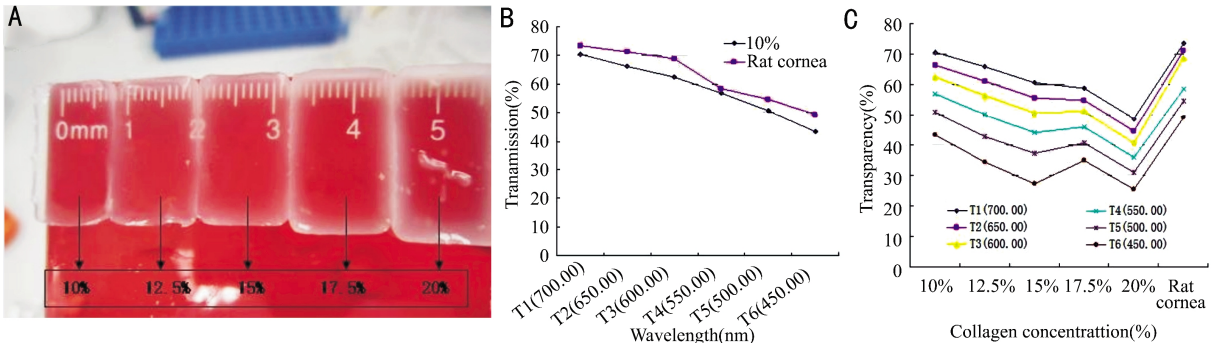

Figure 2 Collagen scaffolds in different concentrations A: Different concentrations; B: 10% collagen concentration group was compared with normal rat cornea; C:The relationships between transparency and collagen concentrations of the collagen implants.

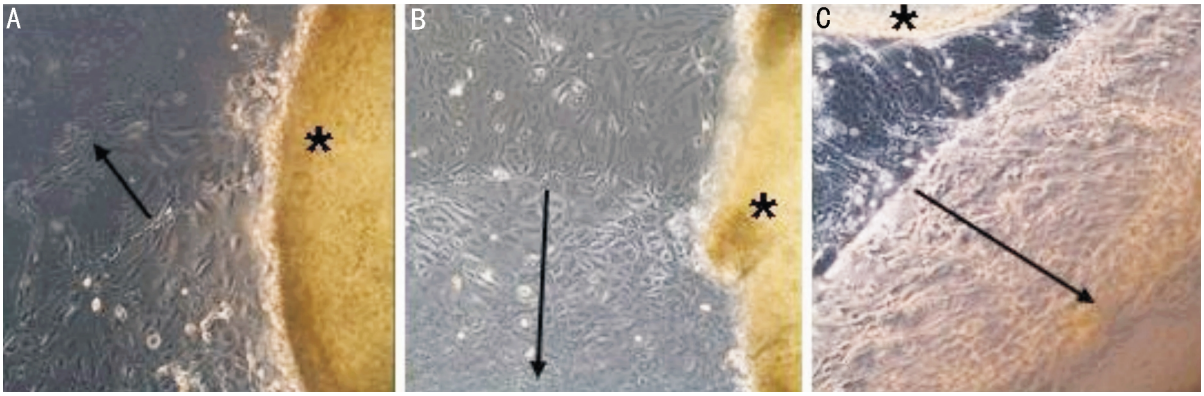

Figure 3 Primary culturing images, inverted microscope A: the first 2 weeks; B, C: 4 weeks; Black arrows indicate the corneal cells growing on the scaffold. * was rat corneal tissue(×200).

Mechanical Properties The groups of higher collagen concentration (20%, 17.5%, 15%) had higher strength than lower groups. The former collagen buttons could be knotted and embedded on the edge by 10-0 sutures under the microscope in vitro without shearing and tearing.

***In vitro* primary corneal epithelial cell culture** In the first 3 weeks, corneal cells proliferation was very active. With the increase of culturing days, the area of corneal cells climbed and growth on the scaffold were larger. The shape of cells which grew on the substitute had no significant differences with the cells before they climbed on the scaffold under the invert microscope (Figure 3). However, on the fortieth day, most cells easily detached from the scaffold and died.

Histopathologic examination of the artificial cornea Reconstructed corneas were fixed with Alcohol-Formalin solution and embedded in paraffin. The 4mm thick sections were stained with hematoxylin-eosin (Figure 4A, B).

DISCUSSION

Corneal transparency is an essential optical property and recreating this aspect is an obstacle to overcome in developing a tissue-engineering scaffold. Results indicate that the 10% collagen concentration group most closely resembled normal rat cornea in its optical properties. Regarding strength for implantation surgery, the group with higher concentrations of collagen had more advantages than the less concentrated groups. This suggests exploring avenues to improve strength in scaffolds with lower collagen

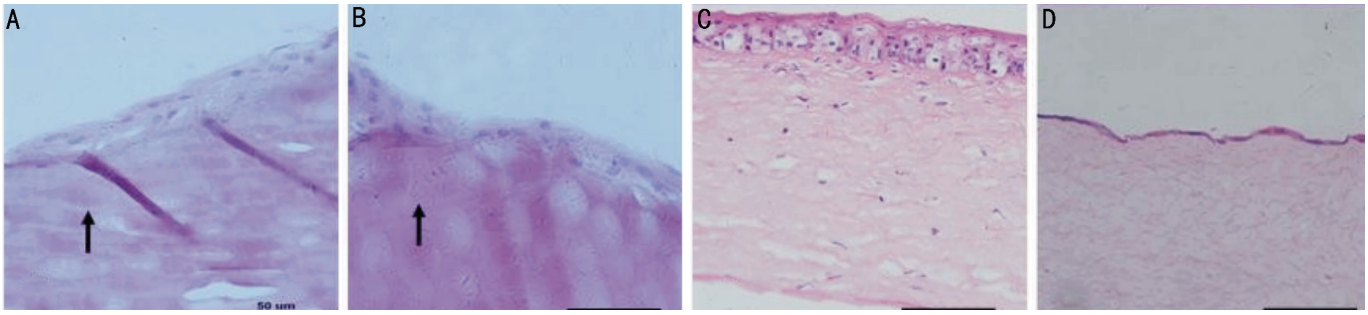

Figure 4 Histological images (×400) A, B: Corneal cells were observed growing as multilayer on the collagen scaffold, black arrows indicate the collagen layer; C: The normal construction of normal rat corneal before cultured on scaffold *in vitro*; D: The rat cornea cultured after 4 weeks.

concentration. The technique of UV-A/Riboflavin on collagen cross-linking[31,32] may be one approach to solve this defect.

The cornea is not void of immune cells. Dendritic and langerhans cells exiting corneal surrounding areas will lead to immune rejection with corneal injury. Consequently, a second problem in developing tissue-engineering cornea relates to its biocompatibility. Unlike the synthetic material, collagen from animal tissues has significant advantages in histocompatibilty. The former studies [33] usually use immortalized corneal epithelial cells which we think may not be safe enough to be used in clinical use, which is a reason to persist in primary culture of corneal cells *in vitro*. As indicated in the images (Figure 3), during the corneal scaffold culturing, the scaffold did not degrade, implying its ability to resist digestive enzymes. In addition, cell shapes that grew on the substitute had no significant difference with the cells before they climbed on to the scaffold. This also suggests the collagen is non-toxic to the cells. In the first 3 weeks, corneal cell proliferation was observed to be highly active. However, cell growth had slowed after the 4th week. On the fortieth day, it was noted that most cells detached from the scaffold and died. Although Ma *et al*[34] developed a reproducible procedure for long-term culturing of corneal epithelial cells from a single rat cornea, the cholera toxin in the culture medium may have played a role and whether it is safe to use in clinical practice would require further confirmation. In this study, growing conditions of corneal cells were structured to resemble *in vivo* settings. Cells which grow on collagen may have originated from normal corneal epithelium as seen in above figures (Figure 4C compared with Figure 4D). The deceleration of cell proliferation sparks an interest in methods to improve corneal epithelial proliferation *in vivo*.

CONCLUSION

Collagen from animal species with chemical cross-linking agents (NHS/EDC) may engineer artificial corneal implants. By adjusting the concentration of collagen, the graft transmittance resembles the cornea of normal rats. The biological stability and biocompatibility of collagen implants from animals demonstrate its potential use to material tissue-engineering corneal scaffold.

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REFERENCES

1 Whitcher JP, Srinivasan M, Upadhyay MP. Corneal blindness: a global perspective. *Bull World Health Organ* 2001; 79(3): 214–221

2 Manuelidis EE, Angelo JN, Gorgacz EJ, Kim JH, Manuelidis L. Experimental creutzfeldt–jakob disease transmitted via the eye with infected cornea. *N Engl J Med* 1977; 296(23): 1334–1336

3 Gomaa A, Comyn O, Liu C. Keratoprostheses in clinical practice – a review. *Clin Experiment Ophthalmol* 2010; 38(2): 211–224

4 Hsiue GH, Lee SD, Chang PC. Surface modification of silicone rubber membrane by plasma induced graft copolymerization as artificial cornea. *Artif Organs* 1996; 20(11): 1196–1207

5 Legeais JM, Drubaix I, Briat B, Renard G, Pouliquen Y. [2nd generation bio–integrated keratoprosthesis. Implantation in animals]. *J Fr Ophtalmol* 1997; 20(1): 42–48

6 Todani A, Ciolino JB, Ament JD, Colby KA, Pineda R, Belin MW, Aquavella JV, Chodosh J, Dohlman CH. Titanium back plate for a PMMA keratoprosthesis: clinical outcomes. *Graefes Arch Clin Exp Ophthalmol* 2011; 249(10):1515–1518

7 Lou X, Dalton PD, Chirila TV. Hydrophilic sponges based on 2–hydroxyethyl methacrylate: part VII: modulation of sponge characteristics by changes in reactivity and hydrophilicity of crosslinking agents. *J Mater Sci Mater Med* 2000; 11(5): 319–325

8 Friend J, Kinoshita S, Thoft RA, Eliason JA. Corneal epithelial cell cultures on stromal carriers. *Invest Ophthalmol Vis Sci* 1982;23(1): 41–49

9 McLaughlin CR, Acosta MC, Luna C, Liu W, Belmonte C, Griffith M, Gallar J. Regeneration of functional nerves within full thickness collagen–phosphorylcholine corneal substitute implants in guinea pigs. *Biomaterials* 2010; 31(10): 2770–2778

10 Deng C, Li F, Hackett JM, Chaudhry SH, Toll FN, Toye B, Hodge W, Griffith M. Collagen and glycopolymer based hydrogel for potential corneal application. *Acta Biomater* 2010; 6(1): 187–194

11 Rafat M, Matsuura T, Li F, Griffith M. Surface modification of collagen–based artificial cornea for reduced endothelialization. *J Biomed Mater Res A* 2009;88(3):755–768

12 Merrett K, Liu W, Mitra D, Camm KD, McLaughlin CR, Liu Y, Watsky MA, Li F, Griffith M, Fogg DE. Synthetic neoglycopolymer–recombinant human collagen hybrids as biomimetic crosslinking agents in corneal

tissue engineering. *Biomaterials* 2009;30(29): 5403-5408

13 Liu W, Deng C, McLaughlin CR, Fagerholm P, Lagali NS, Heyne B, Scaiano JC, Watsky MA, Kato Y, Munger R, Shinozaki N, Li F, Griffith M. Collagen-phosphorylcholine interpenetrating network hydrogels as corneal substitutes. *Biomaterials* 2009;30(8): 1551-1559

14 Nishida K, Yamato M, Hayashida Y, Watanabe K, Yamamoto K, Adachi E, Nagai S, Kikuchi A, Maeda N, Watanabe H, Okano T, Tano Y. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N Engl J Med* 2004;351 (12): 1187-1196

15 Wu Z, Zhou Y, Li N, Huang M, Duan H, Ge J, Xiang P, Wang Z. The use of phospholipase A(2) to prepare acellular porcine corneal stroma as a tissue engineering scaffold. *Biomaterials* 2009; 30(21): 3513-3522

16 Pang K, Du L, Wu X. A rabbit anterior cornea replacement derived from acellular porcine cornea matrix, epithelial cells and keratocytes. *Biomaterials* 2010; 31(28): 7257-7265

17 Du L, Wu X. Development and Characterization of a Full-Thickness Acellular Porcine Cornea Matrix for Tissue Engineering. *Artif Organs* 2011; 35(7):691-705

18 Zhou Y, Wu Z, Ge J, Wan P, Li N, Xiang P, Gao Q, Wang Z. Development and characterization of acellular porcine corneal matrix using sodium dodecylsulfate. *Cornea* 2011; 30(1): 73-82

19 Hashimoto Y, Funamoto S, Sasaki S, Honda T, Hattori S, Nam K, Kimura T, Mochizuki M, Fujisato T, Kobayashi H, Kishida A. Preparation and characterization of decellularized cornea using high-hydrostatic pressurization for corneal tissue engineering. *Biomaterials* 2010; 31(14): 3941-3948

20 Liang Y, Liu W, Han B, Yang C, Ma Q, Zhao W, Rong M, Li H. Fabrication and characters of a corneal endothelial cells scaffold based on chitosan. *J Mater Sci Mater Med* 2011; 22(1): 175-183

21 Minami Y, Sugihara H, Oono S. Reconstruction of cornea in three-dimensional collagen gel matrix culture. *Invest Ophthalmol Vis Sci* 1993; 34(7): 2316-2324

22 Chang JE, Basu SK, Lee VH. Air-interface condition promotes the formation of tight corneal epithelial cell layers for drug transport studies. *Pharm Res* 2000; 17(6): 670-676

23 Mohan RR, Possin DE, Sinha S, Wilson SE. Development of genetically engineered tet HPV16-E6/E7 transduced human corneal epithelial clones having tight regulation of proliferation and normal differentiation. *Exp Eye Res*2003; 77(4): 395-407

24 Robertson DM, Li L, Fisher S, Pearce VP, Shay JW, Wright WE, Cavanagh HD, Jester JV. Characterization of growth and differentiation in a telomerase-immortalized human corneal epithelial cell line. *Invest Ophthalmol Vis Sci* 2005; 46(2): 470-478

25 Ahn JI, Lee DH, Ryu YH, Jang IK, Yoon MY, Shin YH, Seo YK, Yoon HH, Kim JC, Song KY, Yang EK, Kim KH, Park JK. Reconstruction of rabbit corneal epithelium on lyophilized amniotic membrane using the tilting dynamic culture method. *Artif Organs*2007; 31(9): 711-721

26 Carrier P, Deschambeault A, Talbot M, Giasson CJ, Auger FA, Guerin SL, Germain L. Characterization of wound reepithelialization using a new human tissue-engineered corneal wound healing model. *Invest Ophthalmol Vis Sci* 2008; 49(4): 1376-1385

27 Miyashita H, Shimmura S, Higa K, Yoshida S, Kawakita T, Shimazaki J, Tsubota K. A novel NIH/3T3 duplex feeder system to engineer corneal epithelial sheets with enhanced cytokeratin 15-positive progenitor populations. *Tissue Eng Part A* 2008; 14(7): 1275-1282

28 Griffith M, Jackson WB, Lagali N, Merrett K, Li F, Fagerholm P. Artificial corneas: a regenerative medicine approach. *Eye (Lond)* 2009; 23 (10): 1985-1989

29 Duncan TJ, Tanaka Y, Shi D, Kubota A, Quantock AJ, Nishida K. Flow-manipulated, crosslinked collagen gels for use as corneal equivalents. *Biomaterials*2010; 31(34): 8996-9005

30 Duan X, Sheardown H. Dendrimer crosslinked collagen as a corneal tissue engineering scaffold: mechanical properties and corneal epithelial cell interactions. *Biomaterials* 2006;27(26): 4608-4617

31 Snibson GR. Collagen cross-linking: a new treatment paradigm in corneal disease - a review. *Clin Experiment Ophthalmol* 2010; 38 (2): 141-153

32 Zhang Y, Conrad AH, Conrad GW. Effects of ultraviolet-A and riboflavin on the interaction of collagen and proteoglycans during corneal cross-linking. *J Biol Chem* 2011; 286(15): 13011-13022

33 Li F, Carlsson D, Lohmann C, Suuronen E, Vascotto S, Kobuch K, Sheardown H, Munger R, Nakamura M, Griffith M. Cellular and nerve regeneration within a biosynthetic extracellular matrix for corneal transplantation. *Proc Natl Acad Sci U S A* 2003; 100(26): 15346-15351

34 Ma X, Shimmura S, Miyashita H, Yoshida S, Kubota M, Kawakita T, Tsubota K. Long-term culture and growth kinetics of murine corneal epithelial cells expanded from single corneas. *Invest Ophthalmol Vis Sci* 2009; 50(6): 2716-2721